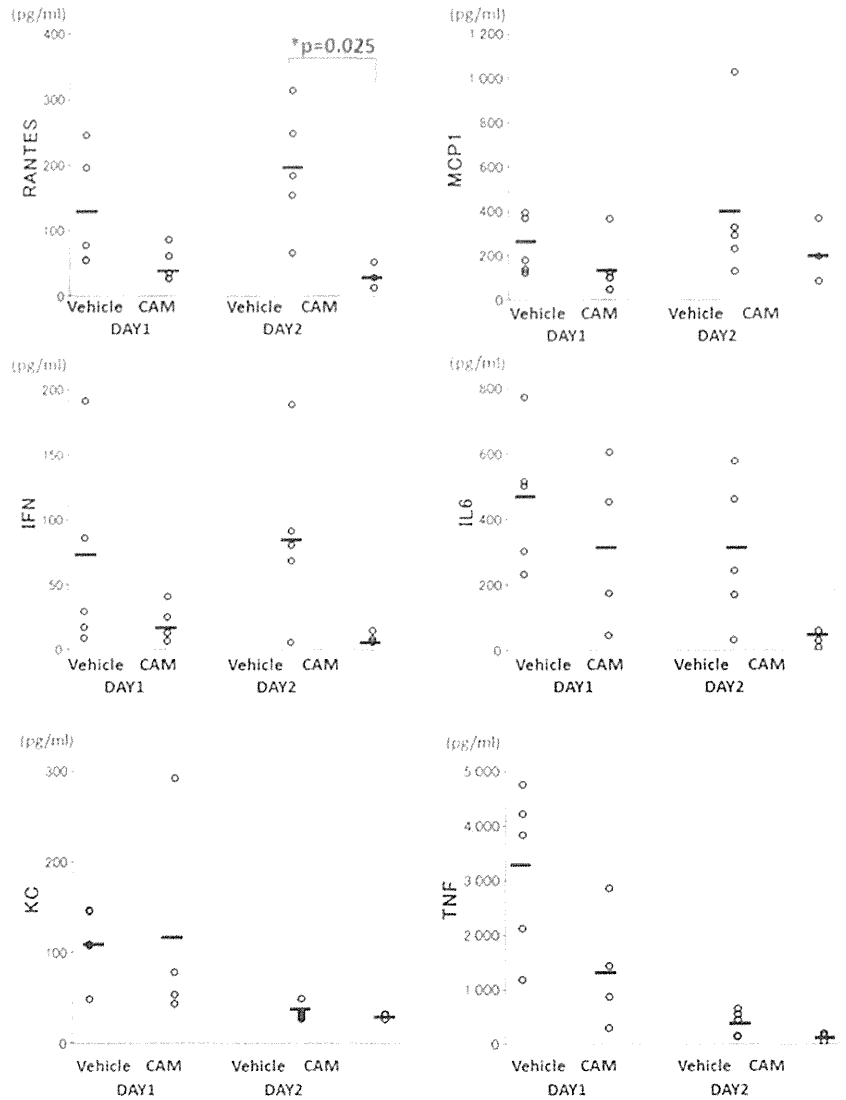

FIGURE 12 | There were fewer cytokines in the CAM50-treated group (50 mg/kg/day orally), compared with the vehicle group. Only RANTES was significantly suppressed in the CAM50 group, at day 2 ($p = 0.025$). BALF: bronchoalveolar lavage fluid. RANTES: regulated on activation, normal T cell expressed and secreted. $^*p < 0.05$.

antigen (day 0) (Saraya et al., 2007a; Saraya and Goto, 2008). Just before and after IT, the 3 groups were orally treated once a day with CAM or vehicle for 2 consecutive weeks. On BAL cell differential count analysis, there were no significant differences in the neutrophil count among the 3 groups in any phase (**Figure 10A**). However, the number of lymphocytes in the CAM-treated groups was significantly suppressed in a dose-dependent manner at day 4, and the effect was still recognized at day 7 (**Figure 10B**). Pathological assessment at day 4 post-IT revealed that the lymphoplasmacytic infiltration within the PBVA was markedly suppressed in the CAM50 group (**Figure 11A**), as compared with that of the vehicle group (**Figure 11B**).

BALF cytokines in the CAM50 group seemed to be lower than those of the vehicle group, and only RANTES was significantly suppressed in the former group, at day 2 ($p = 0.025$) (**Figure 12**). Those data suggested that oral administration of CAM has immunomodulative effects on lung inflammation even in the early phase of Mp pneumonia. This dose-dependent immunomodulative effect of CAM was consistent with previously reported results of a study using an experimental Mp pneumonia mouse model (Tagliabue et al., 2011).

STEROIDS AS ADDITIVE THERAPY

Animal experimental models (Tagliabue et al., 2008; Hirao et al., 2011) showed that corticosteroids down-regulate the host

immune response. Furthermore, treatment with the combination of clarithromycin and a corticosteroid, compared to clarithromycin alone, resulted in a significantly greater reduction of IL-12 p40 and RANTES (Tagliabue et al., 2008). Izumikawa et al. (2014) reported that a majority of human patients with fulminant Mp pneumonia had improved respiratory function on steroid treatment within 3–5 days, which was considered to be an effect of suppressing hyperactivated cellular immunity. Radisic et al. reported on the suppressing effects of steroids on the cell-mediated immune response (Radisic et al., 2000), and that acute respiratory distress syndrome (ARDS) secondary to Mp infection is a lymphoid cellularity ARDS caused by a harmful, "over-reacting" cell-mediated immune response, which could potentially be tapered by the use of steroids. Thus, steroid use would be the preferable treatment of patients with fulminant Mp pneumonia in light of the immune response.

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CASE REPORT

Prenatal molecular diagnosis of X-linked hydrocephalus via a silent C924T mutation in the *L1CAM* gene

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ABSTRACT **We present a case of a patient whose *L1CAM* gene in X-chromosome has a C924T transition. Her first son's ventriculomegaly was prenatally detected. A mature infant was born, his head circumference was large, and thumbs were bilaterally adducted. X-linked hydrocephalus (XLH) was suspected. The DNA examination revealed that both her and boy's *L1CAM* gene had a C924T transition. She became pregnant 5 years later and amniocentesis was performed. The results of cytogenetic analysis revealed that the fetus was female. She continued her pregnancy and delivered a healthy girl. She again became pregnant 3 years later. The chromosomal analysis revealed that the fetus was male. Fetal DNA analysis determined that the fetus had the inherited mutation. She chose to terminate the pregnancy. A C924T mutation can be disease causing for XLH, and the detection of this mutation would aid in genetic counseling for the prenatal diagnosis of XLH.**

Key Words: *L1CAM* gene, prenatal diagnosis, silent mutation, X-linked hydrocephalus

INTRODUCTION

X-linked hydrocephalus (XLH) is one of the genetic forms of hydrocephalus. Mutations in the *L1CAM* gene are now known to be responsible for many cases of XLH. The *L1CAM* gene is located near the telomere of the long arm of the X chromosome and includes 28 exons. L1CAM is a neuronal cell adhesion molecule with important functions in the development of the nervous system. XLH has an incidence of 1/30 000 male births and is characterized by intellectual disability, spastic paraplegia, adducted thumbs, and agenesis of the corpus callosum and/or corticospinal tract (Fernández et al. 2012).

There are no hot mutation spots of *L1CAM* gene. To date more than 200 different mutations have been reported (Vos and Hofstra 2010). These mutations include frameshifts, missense mutations, stop codons, and alterations in splice site junction. A C924T transition is a silent mutation and that was thought to have no effect on the protein sequence. Recently it was reported that this mutation could cause alternative mRNA splicing and congenital hydrocephalus could occur. We present a case of a patient whose *L1CAM* gene has a C924T transition.

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CLINICAL REPORT

A 25-year-old primigravida Japanese woman underwent prenatal management and care at a hospital near her residence. Fetal ventriculomegaly was detected during the 22nd weeks of gestation, so the patient was referred to the obstetrical outpatient clinic of the Niigata University Medical and Dental Hospital for closer examination. The patient had no other known complications or known genetic disease and had conceived spontaneously. Her family history was unremarkable. Her husband was 31 years of age and healthy. She and her husband were not consanguineous.

Fetal biparietal diameter was 60.8 mm and within normal range. A T2-weighted magnetic resonance imaging (MRI) examination revealed severe hydrocephalus and a thinning of the remaining cortex (Fig. 1). Amniocentesis for fetal chromosomal abnormality analysis was performed at the 25th week of gestation. The results of a cytogenetic analysis of cultured amniotic cells revealed a normal karyotype of 46, XY.

Serial ultrasonographic examinations were performed during the pregnancy. As the fetal biparietal diameter gradually enlarged, the patient was hospitalized. She underwent an elective cesarean section at the 38th week of gestation due to the cephalopelvic disproportion. A male infant weighing 2822 g was delivered with an Apgar score of 8 and 9 at 1 and 5 min post-delivery, respectively. His head circumference was 43.5 cm and his thumbs were bilaterally adducted. He received a ventriculoperitoneal shunt at 9 days of age. He had cerebral palsy and subsequently suffered from severe mental retardation.

When the first boy was 3 years old, his head MRI showed hypogenesis of the corpus callosum, thalamus concrescence, and a rippled ventricular wall after shunt placement. The boy's parents were justifiably concerned about the XLH being an inherited disease and asked for a genetic assessment. *L1CAM* gene testing was approved by the Ethics Committees of both the Niigata University and Osaka National Hospitals. The screening was carried out in accordance with the principles of the Declaration of Helsinki, as well as the Ethical Guidelines for Human Genome/Gene Analysis Research required by the Ministry of Education, Culture, Science, and Technology; the Ministry of Health, Labor, and Welfare; and the Ministry of Economy, Trade and Industry of Japan, and by the Guidelines for Genetic Testing in 2003 composed by the genetic medicine-related societies in Japan. After undergoing

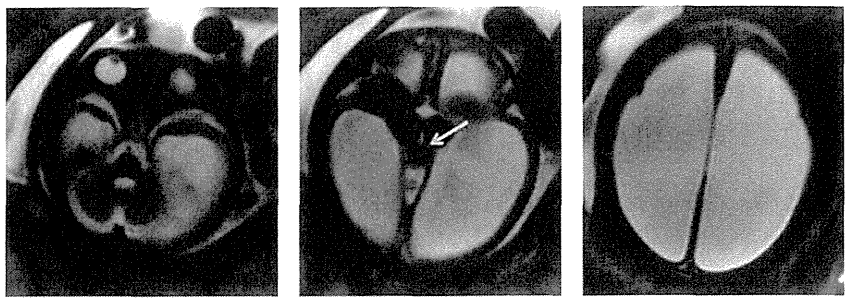

Fig. 1 Fetal magnetic resonance imaging (MRI) at 26th week of gestation. These figures were examined by T2-weighted MRI scan. TR/TE time was 11.90/95 m seconds. It demonstrated dilatation of the lateral ventricles and the third ventricle with aqueductal stenosis. An arrow indicates the aqueductal stenosis.

genetic counseling and having given their informed consent, the parents decided to undertake *L1CAM* gene analysis for their son and themselves. The DNA sequence by the Sanger method (Yamasaki et al. 2011) revealed that the boy's *LICAM* gene had a C924T transition in exon 8. His mother was X-chromosome heterozygous for the same mutation so that she could be a healthy carrier. However, this transition site was initially thought to have no effect on the protein sequence as the alteration affected the third base of a codon (G308G).

The mother and her husband desired to conceive their next baby 4 years after the birth of their first child and requested prenatal genetic testing as part of their genetic counseling. The Niigata University Ethics Committee approved a fetal sex determination by conventional karyotyping for prenatal diagnosis of XLH. We informed the couple that the sex determination could not definitely diagnose their next baby regarding XLH, and that there would be a 50% risk of XLH if the fetus was male and a 50% risk of a healthy carrier if the fetus was female. The woman became pregnant one year later and amniocentesis was performed at the 16th week of gestation. The results of cytogenetic analysis of cultured amniotic cells revealed that the fetus was female (46, XX). She continued her pregnancy and delivered a healthy girl at the 39th week of gestation.

Three years later, she again became pregnant, and requested prenatal genetic testing. Another Japanese family with familial XHL that carried the C924T mutation had been reported (Yamasaki et al. 2011). The Niigata University ethics committee approved a provisional karyotyping analysis for sex determination, and a mutation analysis for the C924T mutation if the fetus was male. At the 16th week of gestation, we obtained amniotic cells by amniocentesis. Fluorescence *in situ* hybridization testing showed that the fetus was in fact male. At the 17th week of gestation, fetal DNA was obtained from amniotic cells and DNA analysis determined that the fetus had inherited the C924T gene mutation. A conventional karyotyping test showed 46, XY. Atrial width was 5.9 mm and no other morphological findings could be detected by ultrasound examination. The mother and her husband chose to terminate the pregnancy at the 18th week of gestation. A stillborn male infant was delivered with a body weight of 240 g and with adducted thumb position. A pedigree of this family is shown in Figure 2.

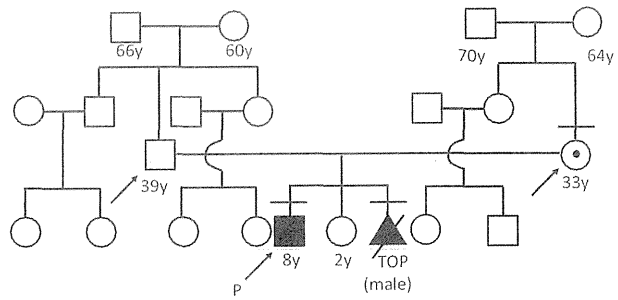

Fig. 2 A pedigree of the patient carrying the L1CAM mutation. A chromosome with the C924T transition was detected in the patient and her two sons. An arrow with P indicates the proband. Only arrow indicates the consultands who are seeking genetic counseling. TOP, termination of pregnancy.

DISCUSSION

XLH has been linked to the genetic L1 syndrome, which encompasses a wide spectrum of diseases, XLH being the most severe phenotype detected *in utero* (Adle-Biassette et al. 2013). The L1 syndrome results from mutations in the *L1CAM* gene located at Xq28 (Yamasaki et al. 1995). *L1CAM* is composed of 28 exons encoding the L1 neural cell adhesion protein, a molecule critical for nervous and enteric system development (Rosenthal et al. 1992). More than 200 *L1CAM* mutations/polymorphisms have been found in unrelated XLH families around the world, of which 51 have been reported in 56 unrelated XLH families within Japan (Yamasaki et al. 2011).

XLH occurs in approximately 5% of all congenital hydrocephalus cases, and the prognosis for XLH is extremely poor (Schrander-Stumpel and Fryns 1998). Neuropathological and molecular data from congenital hydrocephalus cases found that as many as 41% (57/138) had deleterious *L1CAM* mutations (Adle-Biassette et al. 2013). Although it is possible to detect fetal ventriculomegaly by an ultrasound examination, it is extremely difficult to diagnose a congenital hydrocephalus by such means before the 18th week of gestation. In Japan, a termination of pregnancy is legally permitted only before the 22nd week of gestation. Kanemura et al. reported that only 21.7% (5/23) of XLH cases were diagnosed by ultrasound examination prior to the 22nd week of gestation (Kanemura et al. 2006). In our current report of an aborted case, a ventriculomegaly could not be detected at the 18th week of gestation, whereas the XLH-associated *L1CAM* mutation could be definitively detected by a DNA analysis conducted at the 17th week of gestation. Genetic analysis before the 22nd weeks of gestation could thus be very effective for assisting parents in determining whether they should continue a pregnancy or not.

In exon 8, 11 mutation sites were reported according to the database of *L1CAM* gene mutations maintained at the University Medical Center of Groningen web site (http://www.l1cammutationdatabase.info/mutationdetails.aspx). However this is the first report that the C924T transition was prenatally detected in male fetus. The C924T transition in exon 8 occurred in the third base of codon 308, which encodes a glycine, but the change creates

only an amino acid neutral mutation (G308G). Thus, this transition was initially thought to be silent and to have no effect on the protein's sequence. Meanwhile, Du et al. had reported a similar case of XLH with this same "silent" mutation. Du et al. proposed that the mutation could have created a potential 5′ mRNA splice site consensus sequence, which would have resulted in an in-frame deletion of 69 bp from exon 8 and 23 amino acids of the L1CAM protein, although they did not go on to confirm the clinical significance of the C924T mutation (Du et al. 1998). At present, the C924T has been declared as a "disease-causing" site for XLH, according to the above *L1CAM* gene mutation database.

We report on the usefulness of the detection of a familial C924T mutation in the *L1CAM* gene for the prenatal diagnosis of XLH to aid in genetic counseling. In confirmation of this diagnosis, bilateral adducted thumbs were shown after termination at the 18th week of gestation, indicating the likelihood that the fetus had XLH. To the best of our knowledge, this is the first report of a prenatal diagnosis for XLH that was associated with the C924T "silent" mutation. This adds further confirmation that the mutation can be disease causing for XLH.

DISCLOSURE

The authors declare that they have no competing interests.

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Case report

Clinical features of three cases with pulmonary alveolar proteinosis secondary to myelodysplastic syndrome developed during the course of Behçet's disease

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ABSTRACT

We have previously reported that myelodysplastic syndrome (MDS) is the most common underlying disease in cases of secondary pulmonary alveolar proteinosis (PAP). Here, we present 3 MDS cases in which PAP developed during the course of Behçet's disease (BD). All patients carried trisomy 8 in the bone marrow. Chest HRCT scans showed variable distribution of ground glass opacities, but none of the scans showed so called "crazy paving appearance". Two patients with intestinal BD who underwent potent immunosuppressive therapy died of sepsis. These findings demonstrate that PAP secondary to MDS may be occasionally associated with BD.

Abbreviations: BAL, bronchoalveolar lavage; BD, Behçet's disease; GGO, ground glass opacity; HRCT, high-resolution computed tomography; MAC, *Mycobacterium avium complex*; MDS, myelodysplastic syndrome; PAP, pulmonary alveolar proteinosis; RA, refractory anemia; RAEB, RA with excess blasts; SLB, surgical lung biopsy; SPAP, secondary PAP; WLL, whole lung lavage

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1. Introduction

Pulmonary alveolar proteinosis (PAP) is a respiratory disease characterized by accumulation of phospholipids and surfactant proteins within the alveolar lumen and terminal bronchioli. PAP is classified into 3 groups on the basis of etiology: autoimmune PAP (APAP), secondary PAP (SPAP), and unclassified PAP. We previously reported that hematological disorders such as myelodysplastic syndrome (MDS) are the main underlying conditions in SPAP, and are observed in more than 70% of the cases of PAP [1]. However, the precise mechanism underlying the pathogenesis of this condition remains unclear.

Behçet's disease (BD) is a chronic, relapsing, inflammatory disease of unknown etiology that presents with oral aphthae, genital ulcers, uveitis, and skin lesions. Intestinal involvement is seen in 3–26% of cases, with a higher frequency in Asian than European countries. The disease is frequently intractable and may present with severe complications such as hemorrhage, perforation, and infection [2].

In a large cohort study of BD, which included 661 cases, PAP was not reported as a complication [3]. However, 2 BD cases with concomitant PAP have been reported in other studies [4,5]. When we studied the incidence of BD in the 40 Japanese cases of SPAP recorded in our database, we found 5 cases, including the 2 mentioned above. In this report, we present the clinical, radiological, and pathological features of the remaining 3 patients with SPAP and BD, and discuss the characteristic disease features common to all 5 cases, including those previously reported.

SPAP was diagnosed as described previously, with confirmation of the absence of serum GM-CSF autoantibodies [1,6]. BD was diagnosed according to the Behçet Disease Research Committee of Japan criteria [7]. Onset of BD was defined by the emergence of at least 2 symptoms attributable to BD [8]. High-resolution computed tomography (HRCT) images of the chest at the time of diagnosis were evaluated by 2 radiologists (M.A and H.I) as previously reported [6]. Consent was obtained from all identified patients by the treating physicians, and the study was approved by The

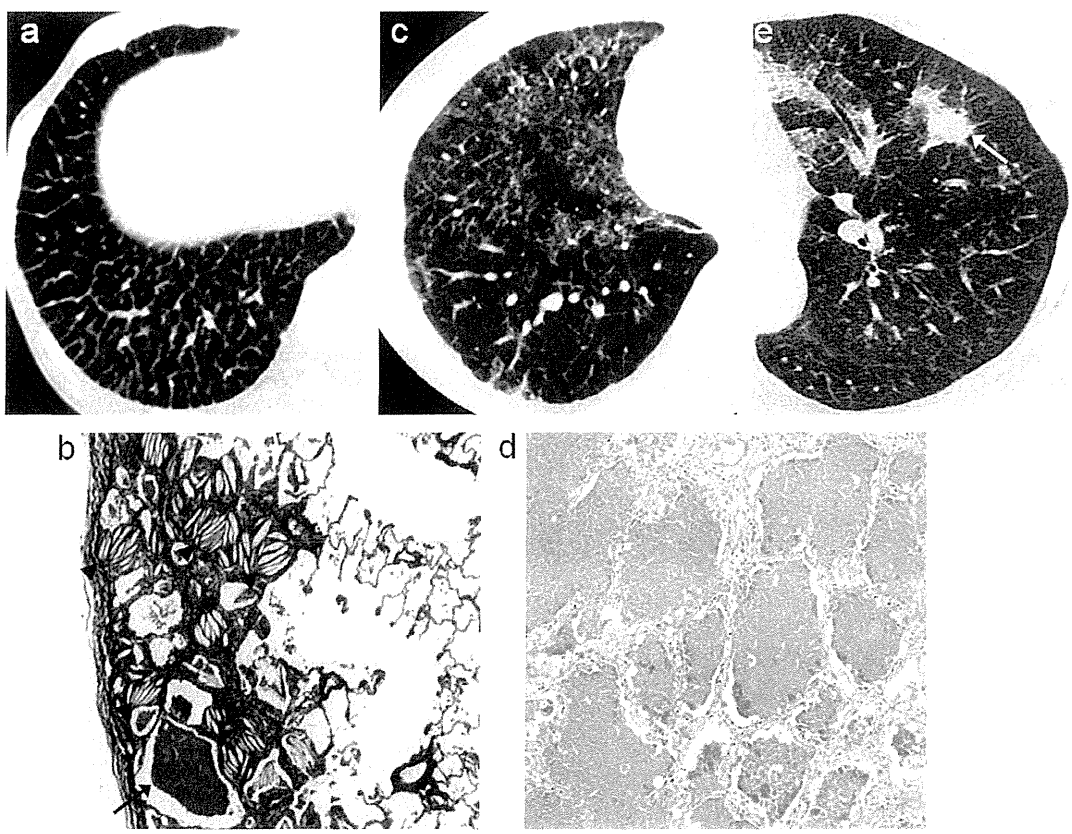

Fig. 1 – (a) Chest HRCT in Case 1 showed diffuse apparent interlobular septal thickening. (b) Lung histology on surgical lung biopsy (SLB) (left S8) in Case 1. Elastica van Giesson stain (EVG), ×40, of the lung biopsy specimen showed interstitial infiltration of inflammatory cells with many cholesterol clefts in the perilobular areas (arrow heads). Amorphous material was found in the alveolar lumen (arrow), although it was not a predominant finding. (c) Chest HRCT in Case 2 showed predominant bilateral diffuse GGO in the upper and middle lobes. (d) Autopsy findings of the lung (PAS stain, ×100) in Case 2 showed diffuse alveolar septal thickening with mild cellular infiltration and with PAS-positive granular material within the alveolar spaces. (e). Chest HRCT in Case 3. Initial HRCT showed bilateral diffuse GGO, and multiple dense opacities developed later (arrow). The dense opacity had resolved with MAC treatment.

Institutional Review Board of Kyorin University School of Medicine (approval number H23-085, October 19, 2011).

2. Case presentation

Case 1 involved a female patient who developed oral and genital ulcers and papulopustular skin lesions at 32 years of age. The patient was diagnosed with intestinal BD 5 years later, and with MDS with refractory anemia (RA) and myelofibrosis at 46 years of age. Intestinal BD was diagnosed and prednisolone treatment was started at a dose of 40 mg/day and tapered to 5 mg/day. At 49 years of age, the patient was diagnosed with PAP on the basis of chest HRCT (Fig. 1a) and typical histologic findings in surgical lung biopsy (SLB) (Fig. 1b). Two years later, she was admitted to the hospital due to an exacerbation of intestinal BD and leukemic transformation of MDS. Despite receiving intensive treatment with steroid pulse, cyclosporine A, and infliximab, the intestinal involvement remained refractory without improvement of persistent peritonitis. During treatment, the patient developed pneumonia caused by methicillin-resistant *Staphylococcus aureus* combined with *Mycobacterium avium complex* (MAC). This led to sepsis, with a fatal outcome 6 months after admission. The severity of PAP remained unchanged during the entire disease course.

Case 2 involved a male patient who developed oral ulcers and papulopustular skin lesions at 26 years of age. The patient was diagnosed with intestinal BD the following year, and MDS (RA) 5 years later. BD was refractory to long-term treatment with prednisolone combined with sulfasalazine. Thereafter, azathioprine combined with etanercept was administered for several weeks, but did not yield any improvement. At 33 years of age, the patient was admitted for treatment of intractable intestinal BD, when he was diagnosed with PAP on the basis of the findings of chest HRCT (Fig. 1c) and BAL. Whole lung lavage (WLL) could not be performed due to persistent fever, although no pathogen was detected. The patient died of PAP deterioration and septic shock 2 months after admission. Autopsy findings revealed

Table 1 – Clinical features of secondary pulmonary alveolar proteinosis complicated with Behçet's disease.

	Case 1	Case 2	Case 3	Literature case 1 [4]	Literature case 2 [5]	Control (n=35)
At diagnosis of PAP						
Age	49	33	50	51	39	53 (24–77)
Gender	F	M	F	F	F	M/F 21/14
Smoking history[a]	NS	S	S	S	S	S/NS/NA 19/11/5
Respiratory symptoms	none	cough	DOE	DOE	cough	16/35 (46%)
Diagnostic procedure	SLB	BS	SLB	BS	BS	BS/SLB 27/8
Serum KL-6 (U/mL)	936	1220	1050	1960	4160	2040 (358–20210)
Serum CEA (ng/mL)	2.0	12.6	NA	3.3	39.5	3.9 (0.5–36.0)
HRCT findings						
GGO pattern[b]	Diffuse	Diffuse	Diffuse	Patchy	Mixed	Diffuse 12/17 (71%)
'Crazy-paving' appearance	–	–	–	+	+	1/17 (6%)
Subpleural sparing	–	–	–	+	–	6/17 (35%)
Thickening of interlobular septa	+	–	–	–	+	4/17 (24%)
Pathological findings	SLB	Autopsy	SLB	N/A	N/A	
Distribution	Perilobular	Diffuse	Patchy			
Cholesterol clefts	Remarkable	Rare	Moderate			
Behçet's disease						
HLA-B51	–	–	N/A	N/A	N/A	
Oral ulcer	+	+	+	+	+	
Eye lesion	–	–	+	–	–	
Skin lesion	+	+	+	+	+	
Genital ulcer	+	–	+	+	+	
Intestinal lesion	+	+	–	–	+	
MDS	+	+	+	+	–	22 (63%)
WHO classification	RA	RA	RAEB-2	RAEB-1		
Trisomy 8	+	+	+	+		5/22 (23%)
WPSS	2	1	3	3		
Duration of BD prior to the onset of MDS	14 Years	5 Years	6 Months	14 Years		
Treatment before MDS onset[c]	P, S, C, T	P, S, A, T	Celecoxib	Colchicine		

Data are expressed as median (range). The control cohorts are patients with secondary pulmonary alveolar proteinosis but without Behçet's disease. NS, never smoked; DOE, dyspnea on effort; BS, bronchoscopy; SLB, surgical lung biopsy; RA, refractory anemia; RAEB, RA with excess of blasts; WPSS, WHO classification-based Prognostic Scoring System (1, low; 2, intermediate; 3, high-risk group); HRCT, high-resolution computed tomography; GGO, ground glass opacity and N/A, not available.

[a] S, current or former smoker.

[b] Mixed, mixed patchy geographic and diffuse pattern; patchy, patchy geographic pattern.

[c] P, prednisolone; S. sulfasalazine;C, cyclosporine A; T, TNF α inhibitors and A, azathioprine.

invasive aspergillosis in the lung, but there was no evidence of leukemic transformation in the bone marrow.

Case 3 involved a female patient who developed uveitis at 38 years of age, which subsequently resolved without treatment. At the age of 48 years, she developed genital ulcers, skin lesions, and oral ulcers, and was diagnosed with complete BD. Six months later, abnormal shadowing was seen on a chest HRCT scan (Fig. 1e), and pancytopenia developed. The patient was diagnosed with PAP and MAC infection by surgical lung biopsy, and MDS (RA with excess blasts [RAEB] type 2) by bone marrow aspiration. Although lung opacity improved with treatment for MAC, the patient showed deterioration of PAP in the subsequent 2 years and required long-term oxygen therapy. The patient underwent WLL and showed improvement of lung opacity and oxygenation.

3. Discussion

In this report, we present 3 patients who developed SPAP during the course of BD and MDS. Their clinical, radiological, and pathological data, as well as those of the 2 cases of SPAP and BD already published in the literature [4,5] and the 35 cases of SPAP without BD in the Japanese SPAP registry [1] are summarized in Table 1. The clinical courses of the 3 patients are shown in Fig. 2. Notably, all 3 patients as well as another patient described in the literature [4] carried trisomy 8 in the bone marrow, and the onset of BD consistently preceded the onset of MDS and SPAP in these patients. HRCT features showed diffuse homogenous patterns in the distribution of ground glass opacity with or without thickening of the interlobular septa, which is characteristic for SPAP [6]. Generally, the clinical, serological, and radiological features of SPAP with BD were similar to those seen in the controls (Table 1). Lung pathology findings were variable with respect to the extent of surfactant accumulation, the amount of cholesterol clefts, and the distribution of lesions. Intestinal BD may be associated with fatal infections due to the use of potent immunosuppressive therapy.

The co-occurrence of BD and MDS has been reported mainly in Japan and Korea [8–16]. These case reports were collected, and the demographic features are summarized in supplementary Table S1. A total of 64 cases of MDS complicated with BD or suspected BD have been reported during the period from 1988 to 2012. The meta-analysis revealed a high frequency of intestinal lesions (66%), trisomy 8 (80%) and a low frequency of ocular lesions (13%). In contrast, a nationwide survey of 3187 BD patients in Japan demonstrated a distinct distribution of intestinal and ocular involvements in 15.5% and 69.1%, respectively [8]. Thus, the disease phenotype of the 5 patients with SPAP and BD (Table 1) is similar to the phenotype of MDS-associated BD described in the literature. This is exemplified by the occurrence of trisomy 8 in the present cases. Since the general frequency of trisomy 8 is only 10–15% in MDS [17], MDS with trisomy 8 is likely to be a risk factor for both BD [9–11] and PAP [4], and they occasionally develop together, as in the 3 cases presented in this report. Immunosuppressive therapy for BD can cause MDS, which may subsequently cause SPAP. However, immunosuppressive drugs had not been administered in both case 3 and the case in the literature [4] until the onset of MDS (Table 1).

Previous large cohort studies have demonstrated a mortality rate of 5–9.8% during a follow up period of 7.7–19 years [18,19]. However, the effect of immunosuppressive treatment or the presence of intestinal lesions on mortality has not been evaluated. Patients with BD complicated with MDS were frequently treated with immunosuppressive drugs (Table S1). Of those, 89% and 94% with and without the intestinal involvements respectively, underwent immunosuppressive

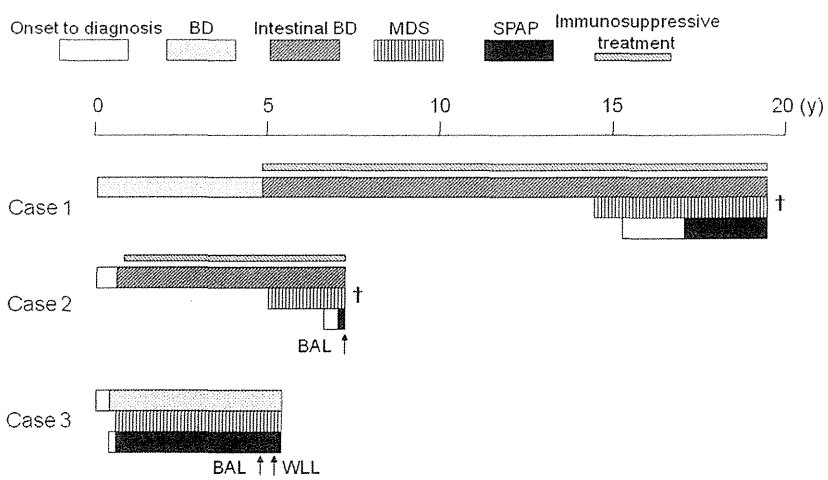

Fig. 2 – Clinical course and prognosis of the patients. Time course of the 3 diseases (BD, Behcet disease; MDS, myelodysplastic syndrome and SPAP, secondary alveolar proteinosis) are shown. Treatments for BD were as follows: prednisolone (5–50 mg/day), sulfasalazine and TNF-α inhibitors in cases 1 and 2, cyclosporine A in case 1, azathioprine in case 2, celecoxib in case 3. Causes of death were as follows: Case 1: leukemic transformation and sepsis due to MRSA and MAC infection; Case 2: sepsis with unknown pathogen and PAP progression.

therapy. It is noteworthy that the mortality rate after such therapy was higher in those with intestinal involvement (41%) than those without involvement (21%). In 5 of 12 deceased cases with intestinal lesions, the cause of death was severe infection. Consistently, 2 fatal cases presented here and another previously published case [5] showed intestinal involvement, and therefore required potent immunosuppressive therapy.

In conclusion, SPAP secondary to MDS is a rare complication during the course of BD. The clinical features other than BD-related findings were not distinguished from those seen in SPAP without BD.

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Conflict of interest

The authors have no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.resinv.2013.05.005.

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Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis

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ABSTRACT

Autoantibodies against granulocyte/macrophage colony-stimulating factor (GMAbs) cause autoimmune pulmonary alveolar proteinosis (PAP) and measurement of the GMAb level in serum is now commonly used to identify this disease, albeit, in a clinical research setting. The present study was undertaken to optimize and standardize serum GMAb concentration testing using a GMAb enzyme-linked immunosorbent assay (GMAb ELISA) to prepare for its introduction into routine clinical use. The GMAb ELISA was evaluated using serum specimens from autoimmune PAP patients, healthy people, and GMAb-spiked serum from healthy people. After optimizing assay components and procedures, its accuracy, precision, reliability, sensitivity, specificity, and ruggedness were evaluated. The coefficient of variation in repeated measurements was acceptable (<15%) for well-to-well, plate-to-plate, day-to-day, and inter-operator variation, and was not affected by repeated freeze–thaw cycles of serum specimens or the reference standards, or by storage of serum samples at −80 °C. The lower limit of quantification (LLOQ) of the PAP patient-derived polyclonal GMAb reference standard (PCRS) was 0.78 ng/ml. Receiver operating characteristic curve analysis identified a serum GMAb level of 5 μg/ml (based on PCRS) as the optimal cut off value for distinguishing autoimmune PAP serum from normal serum. A pharmaceutical-grade, monoclonal GMAb reference standard (MCRS) was developed as the basis of a new unit of measure for GMAb concentration: one International Unit (IU) of GMAb is equivalent to 1 μg/ml of MCRS. The median [interquartile range] serum GMAb level was markedly higher in autoimmune PAP patients than in healthy people (21.54 [12.83–36.38] versus 0.08 [0.05–0.14] IU; n = 56, 38; respectively; $P < 0.0001$). Results demonstrate that serum GMAb measurement using the GMAb ELISA was accurate, precise, reliable, had an acceptable LLOQ, and could be accurately expressed in standardized units. These findings support the use of this GMAb ELISA for the

Abbreviations: PAP, pulmonary alveolar proteinosis; PCRS, polyclonal reference standard; MCRS, monoclonal reference standard; rhGM-CSF, recombinant, human GM-CSF.

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routine clinical diagnosis of autoimmune PAP and introduce a new unit of measure to enable standardized reporting of serum GMAb data from different laboratories.

1. Introduction

Autoimmune pulmonary alveolar proteinosis (autoimmune PAP) is a rare disease characterized by alveolar surfactant accumulation, respiratory failure, and an increased risk of opportunistic infections (Trapnell et al., 2003). The disease is strongly associated with granulocyte/macrophage-colony stimulating factor autoantibodies (GMAbs) (Kitamura et al., 1999) that neutralize GM-CSF bioactivity (Kitamura et al., 1999; Uchida et al., 2004) and mediate pathogenesis by blocking signaling to alveolar macrophages (Sakagami et al., 2009, 2010) and neutrophils (Uchida et al., 2007). Alveolar macrophages require GM-CSF for terminal differentiation and constitutive regulation of functions including surfactant clearance (Shibata et al., 2001; Bonfield et al., 2003). Without pulmonary GM-CSF signaling, alveolar macrophages have impaired pulmonary surfactant clearance (Ikegami et al., 1996) resulting in the slow, progressive surfactant accumulation and the insidious onset of the clinical manifestations of PAP syndrome. Disruption of GM-CSF signaling by recessive mutations in *CSF2RA* or *CSF2RB*, which encode the GM-CSF receptor α and β chains, respectively, causes a hereditary form of PAP that is clinically, physiologically, and histologically indistinguishable from autoimmune PAP (Martinez-Moczygemba et al., 2008; Suzuki et al., 2008, 2010, 2011; Tanaka et al., 2011). PAP can also occur in a heterogeneous group of other diseases either as a consequence of an underlying clinical condition presumably affecting alveolar macrophage function (secondary PAP) or mutations in the genes involved surfactant production (e.g., *SFTPB*, *SFTPC*, *ABCA3*, *TTF1*) (congenital PAP, and PAP associated with interstitial lung disease) (Nogee, 2010).

Clinically, the diagnosis of PAP is made based on a compatible history, typical radiologic findings, and characteristic lung biopsy or bronchoalveolar lavage cytology findings. However, while this approach can determine if PAP syndrome is present, it cannot identify the underlying causative disease. The strong association of a high serum GM-CSF autoantibody (GMAb) level with autoimmune PAP (Kitamura et al., 1999; Bonfield et al., 2002; Trapnell et al., 2003; Inoue et al., 2008), development of an ELISA to measure GMAbs (Schoch et al., 2002), and demonstration that GMAbs actually drive the pathogenesis of autoimmune PAP (Uchida et al., 2007; Sakagami et al., 2009, 2010), support what is now widespread use of serum GMAb measurement for the clinical research diagnosis of autoimmune PAP. The potential clinical use of the GMAb ELISA is further supported by the identification of critical threshold of serum GMAb that is associated with an increased risk of autoimmune PAP (Bendtzen et al., 2007; Uchida et al., 2009; Sakagami et al., 2010). Several GMAb ELISA-based methods have been developed for measurement of serum GMAb including a quantitative method based on use of a neutralizing, polyclonal GMAb reference standard (PCRS) (Kitamura et al., 1999; Schoch et al., 2002) and a non-neutralizing, monoclonal GMAb reference standard (MCRS) (Inoue et al., 2008) that return values in units of GMAb concentration and a serum titration method that returns values in units of GMAb titer (Kitamura et al., 1999; Bonfield et al., 2002).

The purpose of the present study was to optimize the GMAb ELISA with respect to reagents, experimental protocol, and analysis methods, and then validate it by rigorously establishing its sensitivity, accuracy, precision, and ruggedness to support its clinical use for the diagnosis of autoimmune PAP. Further, we compared the relative performance of a GMAb polyclonal reference standard (PCRS) purified from PAP patient serum against a GMAb monoclonal reference standard (MCRS) prepared under good manufacturing practices for potential use as a reference standard for calibrating the results obtained in different laboratories.

2. Materials and methods

2.1. Participants

The institutional review board of the Cincinnati Children's Hospital Medical Center approved the study. All participants or their legal guardians gave written informed consent and minors gave assent. Participants included patients with autoimmune PAP (n = 96; 45.8% male; 37.3 ± 15.7 years of age at evaluation) referred for evaluation or treatment. The diagnosis of autoimmune PAP was based on clinical and radiographic findings; an open lung biopsy, transbronchial lung biopsy, or cytologic analysis of bronchoalveolar lavage cells and fluid; and a positive GMAb test performed as previously described (Uchida et al., 2004). We also studied healthy people (n = 58; 22.4% male; 30.6 ± 7.0 years of age at evaluation) who were nonsmokers with no history of major illness and symptom-free at the time of evaluation.

2.2. GMAb polyclonal reference standard

A GMAb polyclonal reference standard (PCRS) was prepared as previously described (Schoch et al., 2002) from a patient with autoimmune PAP (Luisetti et al., 2009). Briefly, GMAb was isolated from plasmapheresis fluid by protein G column chromatography followed by GM-CSF affinity chromatography (Schoch et al., 2002), concentrated by ultrafiltration (Ultra-15; MWCO 30 kDa, Amicon), and re-suspended in phosphate-buffered saline (PBS). The concentration of GMAb was determined previously by comparison to a GMAb reference standard for which the IgG content had been determined by ELISA (Kitamura et al., 2000). The purity of the PCRS was assessed by polyacrylamide gel electrophoresis (ReadyGel®, 5–15% gradient, BioRad Laboratories, Hercules, CA) with 2-mercaptoethanol. A 'PCRS Master Stock' containing

the final purified GMAb at 2 mg/ml in PBS was prepared as 10 µl aliquots in 0.5 ml polypropylene tubes (Eppendorf, Hamburg, Germany) and stored at −80 °C with continuous electronic temperature monitoring as previously reported (Uchida et al., 2009). A 'PCRS Working Standard' was prepared by diluting one vial of PCRS Master Stock with ELISA dilution buffer to GMAb concentration of 100 ng/ml and stored as 250 µl frozen aliquots as above. GMAb ELISA plate concentration standards were prepared from the PCRS Working Standard by thawing one vial at room temperature and diluting a 120 µl aliquot serially (1/2) to create standard concentrations (50, 25, 12.5, 6.25, 3.125, 1.5625 ng/ml). ELISA buffer without PCRS served as a 0 ng/ml control. A serial dilution to 0.78125 ng/ml was used in some experiments to define the lower limit of quantification (LLOQ).

2.3. GMAb monoclonal reference standard

A GMAb monoclonal reference standard (MCRS) was previously prepared from a PAP patient-derived, Epstein–Barr virus transformed, immortalized B lymphocyte clone. One of six initial cell clones was used to produce the MCRS by Boehringer Ingelheim pharmaceuticals (Biberach, Germany). Briefly, the B cell clone-derived cDNA was used to stably-transduce Chinese hamster ovary D644 cells (Urlaub et al., 1983). A pool of MCRS-producing cells was subjected to 10 day fed-batch cultures in a 30 and 50 l bio-fermenter and MCRS was purified from culture supernatant using protein A chromatography (MabSelect SuRE™, GE Healthcare, Inc.). Purified MCRS was re-suspended in 25 mM sodium citrate, pH 6.0, 115 mM sodium chloride, 0.01% (w/v) polysorbate 20, pH 6. Its binding affinity for GM-CSF was determined to be 423 pM using KinExA (Sapidyne Instruments, Inc.) equilibrium experiments, which were conducted at room temperature (~21 °C). The antibody concentration was held constant as the antigen (rGM-CSF, Biomol GmbH, Germany) was titrated in a 2-fold serial dilution. Solutions were allowed to come to equilibrium prior measurement and the free antibodies were captured by rGM-CSF-rabbit Fc coupled to beads. The final MCRS preparation (monoclonal, non-neutralizing, human anti-human GM-CSF IgG_1 lambda antibody; BI 01049904, Lot 1, 05.08.2009; 500 µg/500 µl; MCRS Original Stock) was shipped on dry ice with continuous electronic temperature recording to Cincinnati, Ohio. A "MCRS Master Stock" was prepared by thawing the Original Stock tube at room temperature, mixing thoroughly by gentle inversion, dispensing as 25 µl aliquots without dilution in 0.5 ml polypropylene tubes (Eppendorf, Hamburg, Germany) and then storing frozen as for the PCRS. A 'MCRS Working Stock' was prepared by diluting one vial (25 µl) of MCRS Master Stock with ELISA dilution buffer (4.975 ml; PBS, Tween-20, 1% BSA) to a final concentration of 5 µg/ml, which was dispensed as 200 µl aliquots and stored frozen as above. A 'MCRS Working Standard' was prepared by diluting one vial (200 µl) of MCRS Master Stock with ELISA dilution buffer (49.8 ml) to 20 ng/ml and stored frozen in 250 µl aliquots as above. GMAb ELISA plate concentration standards were prepared from the MCRS Working Standard as above to create standard concentrations (20, 10, 5, 2.5, 1.25, 0.63, 0.31). ELISA buffer without MCRS serves as the 0 ng/ml control.

2.4. GM-CSF capture antigen

Preparations of recombinant, human GM-CSF from several suppliers (Miltenyi Biotech, Auburn, CA, USA; Prospec-Tany Technogene Ltd, Rehovot, Israel, USA; Invitrogen Corp., Camarillo, CA, USA; Genway Biotech, Inc., San Diego, CA, USA; Genzyme, Cambridge, MA, USA) were evaluated as a capture antigen in the GMAb ELISA by measuring the baseline optical absorbance for each preparation in the absence of added serum sample. The rhGM-CSF from Miltenyi Biotech (Fig. 1C, Supplier 2) was chosen for the standard GMAb ELISA and was used throughout this study except where indicated.

2.5. Human immunoglobulin G detection antibody

Two anti-human immunoglobulin G (IgG) reagents (goat anti-human IgG-specific $F(ab')_2$ antibody fragment, #A2290; and intact goat anti-human-IgG-Fc specific antibody, #A6029; both from Sigma-Aldrich, St. Louis, MO, USA) were evaluated as the secondary detection antibody in the GMAb ELISA. The $F(ab')_2$ antibody was chosen for the standard GMAb ELISA and was used throughout this study except where indicated.

2.6. GMAb ELISA

The standard GMAb ELISA used was based on an initial description (Nakata, 1999; Kitamura et al., 2000; Schoch et al., 2002) and subsequent modifications (Uchida et al., 2004) and performed as follows. Microtiter plates (96-well, Maxisorp; Nalge Nunc International, Rochester, NY) were incubated (4 °C, overnight) with 50 µl of capture antigen solution (1 µg/mL rhGM-CSF (Miltenyi except where noted) in phosphate buffered saline (PBS)), washed in PBS containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), and incubated (room temperature, 1 h) with blocking solution, Stabilcoat® (Surmodics, Eden Prairie, MN, USA). Serum samples (100 µl except where indicated) were mixed with sample dilution buffer (PBS, 1% [vol/vol] BSA, 0.1% [wt/vol] Tween 20) to prepare a standard dilution series (1/101, 1/3001, 1/6001 and 1/12,001) for each sample. Aliquots (50 µl) of diluted serum or reference standards were pipetted into adjacent microtiter wells, incubated (room temperature, 40 min) to allow binding of GMAb to the capture antigen, and then washed five times with 300 µl of wash buffer (PBS, 0.1% [vol/vol] Tween 20). Horse radish peroxidase-conjugated anti-human IgG $F(ab')_2$ fragment (except where noted) was diluted 1/3000 with sample dilution buffer for use as the detection antibody and 50 µl was pipetted into each well. The plates were incubated (room temperature, 30 min) to allow binding of IgG detection antibody to captured GMAb and then rinsed 4 times with wash buffer. Chromogenic substrate solution (50 µl; 3,3′,5,5′-tetramethylbenzidine; T4444, Sigma-Aldrich) was added to each well and the plates were incubated (room temperature, 15 min) to permit color development, which was stopped by adding 50 µL of 1 N H_2SO_4. Optical absorbance at wavelength of 450 nm was measured using a Benchmark® ELISA plate reader using Microplate Manager software, version 5.21 (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufacturer.

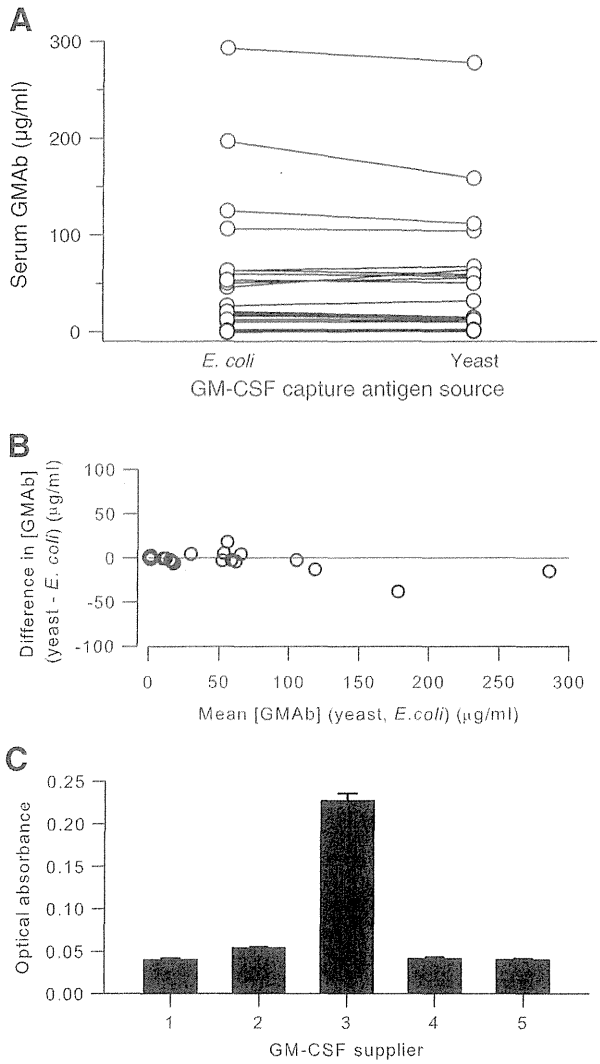

Fig. 1. Effects of the capture antigen on GMAb ELISA performance. A. Non-glycosylated versus glycosylated GM-CSF capture antigen. Serum GMAb concentration measured with the GMAb ELISA using GM-CSF produced in *E. coli* (non-glycosylated form) or in yeast (glycosylated form). Each symbol represents the mean of 2 determinations on serum samples from each autoimmune PAP patient or healthy person (n = 20 and 10, respectively). The GMAb concentration measured for each sample with each capture antigen was not different (P = 0.796, n = 30; Mann–Whitney Rank Sum Test). B. Bland and Altman analysis. All data shown in panel A were evaluated by Bland and Altman analysis as described in the Materials and methods section. C. Source of *E. coli*-derived GM-CSF capture antigen. ELISA plates were coated with GM-CSF (1 μg/ml) produced in *E. coli* obtained from several suppliers (1–5) and then used in the GMAb ELISA with sham samples (i.e., buffer instead of serum) to determine the effect on background optical absorbance. Each bar represents the mean (±SD) of 4 separate determinations. The background absorbance was different for all comparisons except between suppliers 1, 4, and 5 (P < 0.001, n = 4 each; ANOVA with pairwise multiple comparison procedures by the Holm–Sidak method).

GMAb concentration was determined as follows. The mean optical absorbance of the PCRS in replicate wells was plotted (x-axis) against the known GMAb concentration (y-axis) and quadratic regression analysis (except where noted) was performed to determine the equation relating optical absorbance to PCRS concentration (ng/ml on plate) using Microsoft Excel (Microsoft Corp, Seattle, WA, USA). The mean optical absorbance of replicate wells for each sample was substituted for x in the regression equation to determine the concentration of GMAb in the well in ng/ml, which was multiplied by the dilution factor to determine the GMAb concentration in serum in μg/ml.

Quality control standards consisting of duplicate serum samples of known GMAb concentration were run on every plate. All results from any plate for which the quality control replicates differed by more than 15% were rejected, which occurred in one of eighty plates in this study.

2.7. Statistical analysis

Numerical data were evaluated for a normal distribution using the Shapiro–Wilk test and for equal variance using the Levene median test. Parametric data are presented as means (±SD) and nonparametric data are presented as medians (interquartile range [IQR]). Statistical comparisons of parametric data were made with Student's *t*-test for two-group comparisons and with one-way analysis of variance with post hoc analysis by the Holm–Sidak method for comparisons among three or more groups. Nonparametric data were compared with the use of the Mann–Whitney Rank-Sum test. Agreement between assay methods was made using Bland–Altman difference analysis. Receiver operating characteristic (ROC) curve analysis and other statistical analyses were done using SigmaPlot software (Version 12.3, Systat Software, San Jose, CA, USA). P values less than 0.05 were considered to indicate statistical significance. All experiments were repeated at least twice, with similar results.

3. Results

3.1. Optimization of GMAb ELISA components and procedure

GMAb concentration measured with the GMAb ELISA using *E. coli*- or yeast-derived GM-CSF as the capture antigen was similar indicating that glycosylation had little effect on capture antigen function (Fig. 1A, B). Because prior studies indicated that some rhGM-CSF preparations had a high background absorption, GM-CSF from multiple sources was evaluated. Background absorbance in the GMAb ELISA varied significantly for rhGM-CSF from different suppliers (Fig. 1C). Thus every batch of rhGM-CSF considered for use as the capture antigen must be checked for acceptability to ensure a low background absorbance since higher values will affect the cutoff between normal and disease (see below). Non-glycosylated rhGM-CSF produced in *E. coli* was chosen for use in the standard GMAb ELISA to minimize potential non-specific binding to the carbohydrate moiety.

Use of an $F(ab')_2$ fragment (#A2290) or intact IgG (#A6029) as the anti-human IgG secondary (detection) antibody in the GMAb ELISA gave similar results except at higher GMAb concentrations where the intact IgG gave slightly higher values (Fig. 2A, B). Since $F(ab')_2$ gave good results and was used in our prior reports (Schoch et al., 2002; Trapnell et al., 2003; Uchida et al., 2004, 2007, 2009), it was chosen for routine use in the standard GMAb ELISA to maximize comparability to previously reported results.

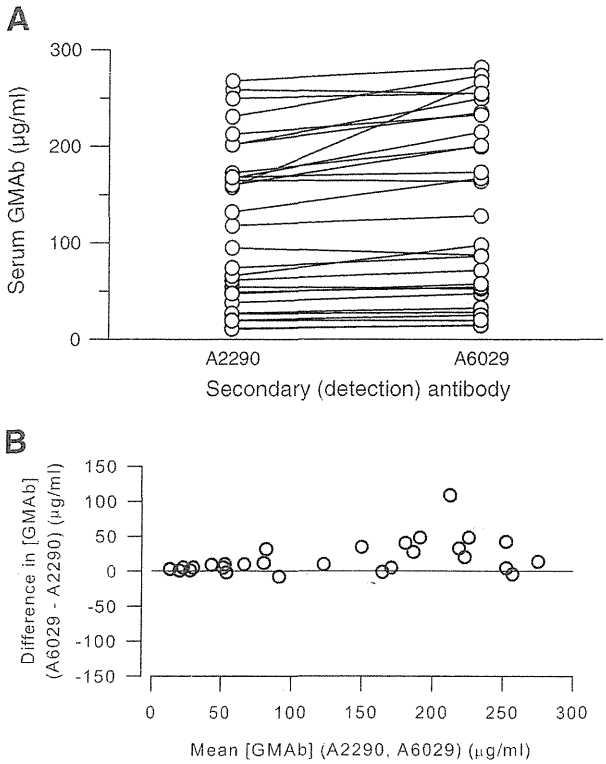

Fig. 2. Effects of the secondary detection antibody on GMAb ELISA performance. A. Use of intact goat-anti-human IgG or an $F(ab')_2$ fragment as the detection antibody. Serum GMAb concentration measured with the GMAb ELISA in parallel using either an $F(ab')_2$ fragment (A2290) or intact anti-human IgG (A6029) as the detection antibody. Each symbol represents the mean of 3 determinations on the serum sample from a different autoimmune PAP patient. The median GMAb concentration (not shown) determined with the intact IgG secondary antibody was slightly higher than with the $F(ab')_2$ secondary antibody (128 [51–234] vs 118 [43–187] μg/mL; $P < 0.001$; $n = 29$ serum samples; Wilcoxon Signed Rank test). B. Bland and Altman analysis. All data shown in panel A were evaluated by Bland and Altman analysis as described in the Materials and methods section.

Patient-derived GMAb PCRS contained three molecular species similar in molecular mass to IgG1, IgG2, (predominant species of roughly equal proportion) and IgG3, and IgG4 (minor species; 0.2–3.5%) (Fig. 3A) consistent with our prior report that GMAbs from autoimmune PAP patients and healthy people are composed primarily of IgG1 and IgG2 with negligible amounts of IgG3 and IgG4 (Uchida et al.,

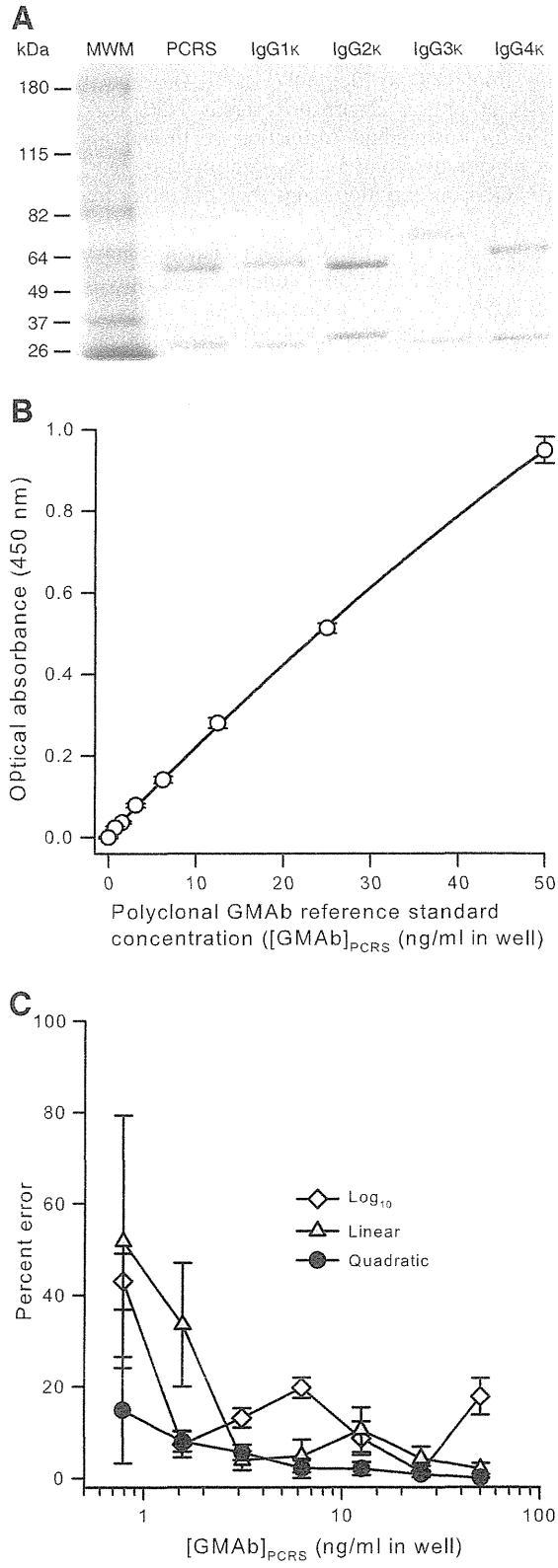

Fig. 3. Characterization and performance of a PAP patient-derived GMAb polyclonal reference standard (PCRS). A. Purity and composition of the PCRS. PCRS, prepared as described in the Materials and methods section, commercially available IgG heavy chain isotype standards (IgGκ 1, 2, 3, or 4), or molecular weight markers (MWM) were subjected to polyacrylamide gel electrophoresis under reducing conditions, Coomassie blue staining, and photography as described in the Materials and methods section. B. Optical absorbance of the PCRS as a function of concentration. The PCRS was serially diluted and evaluated as the standard in the GMAb ELISA as described in the Materials and methods section. Optical absorbance increased smoothly in proportion with PCRS concentration. Regression analysis using a quadratic equation yielded a correlation coefficient (R^2) of 0.9998. Each point represents the mean (+SD) of 5 separate measurements. C. Effect of regression method used on percent error of the PCRS curve rit. Results from 5 independent, simultaneously conducted experiments determining the optical absorbance of serial dilutions of the PCRS were subjected individually to linear, quadratic, or logarithmic regression analysis and the mean (±SD) percent deviation at each concentration was determined. The percent error of GMAb concentration was determined as the measured value minus the expected value divided by the expected value and multiplied by 100. The mean (±SD) correlation coefficients for regression analysis of 5 separate experiments (not shown) were 0.9999 ± 0.0001 (quadratic), 0.9969 ± 0.0029 (log_{10}), and 0.9819 ± 0.0043 (linear).